REMARKS

At the outset, the undersigned would like to thank the Examiner for the courtesies extended during the telephonic interview on November 17, 2005 between the Examiner, the undersigned and Dr. Sam Gandy, and on November 21, 2005, between the Examiner and the undersigned. During the interviews, the rejection under the enablement requirement and the rejections under 35 U.S.C. §102(b) and 35 U.S.C. §103 were discussed. In addition, proposed claim amendments, which are set forth in the instant amendment, were discussed with the Examiner.

Claims 1-33 are currently pending in this application. Claims 20, 22, and 23 have been amended and claims 21, 32, and 33 have been canceled, without prejudice. Claims 7-19 and 26-30 were directed to a non-elected invention and have been canceled without prejudice. Thus, upon entry of this amendment, claims 1-6, 20, 22-25, and 31 will be pending in the instant application. Support for the amendments to claims 20, 22, and 23 can be found in the specification at least, for example, page 8, lines 18-22 of the specification. No new matter has been added.

Rejections under 35 U.S.C. §112, First Paragraph

The Examiner has rejected claims 1-2, 4-6, 20-25, and 31-33 as allegedly lacking enablement. The Examiner states that "treatment with physiological levels of estradiol in vitro results in large increases in soluble APP and according to applicant that [*sic*] observing no effect on this soluble APP levels is surprising after estradiol administration." (Office Action, page 2). The Examiner contends that the specification describes "multitudes of compounds having steroidal structure" which fall within the generic term "estrogen compound" and that the specification does not contain evidence that *in vivo* administration of any compound falling within this generic term would lead to the same surprising results.

The Examiner also contends that there is no adequate guidance in the specification to determine amounts of compounds which would not have an effect on the soluble APP but would decrease the levels of beta peptides. Further, the Examiner contends that there is no evidence as to how one can predict the susceptibility of a human to Alzheimer's disease and how the treatment of

these people with estrogens would delay or reduce the likelihood or ameliorate Alzheimer's disease, and other diseases wherein amyloidosis is involved. The Examiner also states that it would require undue experimentation to determine which of the compounds falling within the definition of estrogen compound would have the effect of estradiol on amyloid beta peptide levels without having an affecting soluble APP.

The Examiner's rejection is respectfully traversed. Claim 20 has been amended to refer to a method for delaying or reducing the likelihood of, or ameliorating, a disease or disorder associated with amyloidosis, which method comprises administering an Aβ level reducing dose of ***17β-estradiol*** to a subject who has an increased risk for developing or shows a symptom of the disease or disorder associated with amyloidosis, wherein the dose of 17β-estradiol does not affect soluble APP levels. Claim 1 is directed to methods for reducing a level of amyloid-β (Aβ) peptides *in vivo*, comprising administering an Aβ level reducing dose of an estrogen compound to an animal, wherein the animal has an increased level of Aβ, and wherein the dose of the estrogen compound does not affect soluble APP levels.

Applicants respectfully submit that the specification contains working examples describing *in vivo* administration of 17β-estradiol to ovariectomized (ovx) animals and the resulting reduction in Aβ levels without a decrease in soluble APP. With respect to the Examiner's contention that the specification does not contain evidence that *in vivo* administration of any estrogen compound would lead to the same surprising results, Applicants note that as of November 5, 1999, the earliest priority date of this application, a person of ordinary skill in the art would have been able to determine which compound acted as an estrogen compound, and, based on the teachings in the specification, which of such estrogen compounds would reduce Aβ without reducing soluble APP *in vivo,* as claimed, without undue experimentation. The level of soluble APP *in vivo* is easily determined, as described in the specification. Applicants' specification discloses methods for testing estrogen compounds and for determining the effective amounts of estrogen compounds. As set forth in the previous Response (dated March 14, 2005), Example 3 discloses that "[t]he ovariectomized guinea pig model described in Example 1 or the overiectomized transgenic rodent model described in Example 2 can be used to screen for compounds or, more optimally, to evaluate candidate compounds obtained from screens for the ability to affect Aβ levels

in the brains of these animals." See the specification at page 29, ll. 7-11. A person of skill in the art, armed with the knowledge of these models, could easily screen for and identify estrogen compounds useful in the method of the invention, *e.g.*, estrogen compounds that decrease the levels of β peptides, but that do not affect soluble APP (sAPP).

For example, a candidate estrogen compound can be administered to overiectomized guinea pigs. After this treatment, brains can be collected from sacrificed animals for quantification of the Aβ40, Aβ42, and sAPPα levels using ELISA assays and quantitative immunoblotting, respectively. While such experimental procedures might be laborious, they do not constitute undue experimentation. As described in the M.P.E.P., §2164.01, "[t]he fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation," citing *In re Certain Limited-Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). See also *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404. These experiments involve routine techniques (animal models, tissue extraction from sacrificed animals, and well-known immunological and biochemical assays). The Examiner has provided no evidence supporting undue experimentation.

Furthermore, as of the priority date of the instant application, it would not have required one of ordinary skill in the art undue experimentation to predict the susceptibility of a human to Alzheimer's disease. As set forth in the specification, "reduction in the levels of an estrogen compound in vivo results in increased amyloid production. This observation establishes the ability to predict whether a given subject will have an increased likelihood of developing amyloid deposits, and thus an increased likelihood of developing a disease or disorder associated with amyloidosis, *e.g.*, Alzheimer's Disease." (page 14, lines 13-17 of the specification).

With respect to the Examiner's contention that it would require undue experimentation to determine how the treatment of people with estrogen compounds would delay or reduce the likelihood or ameliorate Alzheimer's disease and other diseases wherein amyloidosis is involved, Applicants respectfully submit that, as set forth above, the instant specification describes animal models wherein administration of an estrogen compound reduced levels of Aβ and did not affect

soluble APP levels. As set forth in the Declaration under 37 C.F.R. §1.131, filed on August 10, 2005 ("the August 10, 2004 Declaration)

> [i]t is well known by persons having ordinary skill in the art that animal models can be used to determine the pharmacology of Alzheimer's disease (AD). AD is characterized by the accumulation of, *inter alia*, amyloid plaques and deposits, of which Aβ is a major component (See specification of the '466 application, p. 1, lines 11-20). Aβ is derived by proteolytic processing of APP. *Id.* Guinea pigs are a useful animal model because their endogenous amino acid sequence of the Aβ peptide is identical to the human sequence. *See* Johnstone, et al., "Conservation of the sequence of the Alzheimer's disease amyloid peptide in dog, polar bear and five other mammals by cross-species polymerase chain reaction analysis," Mol. Brain Res. 1991 Jul.; 10(4): 299-305 at 303 (Exhibit 12). Certain transgenic animals are also useful animal models because after introduction of a transgene, they too will express an amino acid sequence of the Aβ peptide identical to the human sequence. Accordingly, the testing described herein supports use of the claimed invention in humans. (paragraph 14 of the August 10, 2004 Declaration).

Furthermore, during the telephone interview of November 21, 2005, the Examiner indicated that prevention of a disease or disorder associated with amyloidosis was not enabled by the specification. The Examiner stated that submission of evidence that administration of an estrogen compound may be used to prevent Alzheimer's disease may overcome the rejection.

Applicants respectfully submit that the specification enables delaying or reducing the likelihood of a disease or disorder associated with amyloidosis as set forth in claim 20. Furthermore, as evidence of the ability of an estrogen compound to prevent a disease or disorder associated with amyloidosis, *e.g.,* Alzheimer's disease, Applicants submit herewith a copy of Zandi et al. ((2002) *JAMA* Vol. 288, No. 17; copy attached as Exhibit 2). Zandi et al. establish that hormone replacement therapy (HRT), including estrogen, is now known in the art to be useful in the prevention of Alzheimer's disease. The findings of Zandi et al., as well as the findings of two previous studies cited by Zandi et al.[1], show that HRT may be useful in the prevention of AD. In

[1] The studies referenced by Zandi et al. as references 16 and 17 (Tang et al. *Lancet* 1996;348:429-432 and Kawas et al. *Neurology* 1997;48:1517-1521) are of record in this application. Zandi et al. also cite additional references with "mixed results" including references 14 and 15 (Brenner et al. *Am J. Epidemiology* 1994;140:262-267 and Waring et al.

particular, Zandi et al. state that these studies "provide new evidence to suggest a protective effect of HRT. As in the previous studies, the adjusted risk of incident AD among lifetime HRT users was reduced to little more than half that among non-users" (Zandi et al., p. 2127). Furthermore, Zandi et al. conclude that their findings "suggest that HRT may be effective for the primary prevention of AD - if not for its treatment. . ." (Zandi et al., p. 2129). Thus, Zandi et al. provide evidence for the ability of estrogen compounds to prevent diseases or disorders associated with amyloidosis, *e.g.*, Alzheimer's disease. Accordingly, the claimed methods for delaying or reducing the likelihood of a disease or disorder associated with amyloidosis are supported by Zandi et al.

In view of the above, Applicants submit that the invention as set forth by the pending claims is enabled, and respectfully request reconsideration and withdrawal of this rejection.

Rejections under 35 U.S.C. §102(b)

The Examiner has maintained the rejection of claims 20, 21 and 23-25 as allegedly being anticipated by Washburn (U.S. Patent No. 5,719,137, referred to herein as "the '137 patent"). In particular, the Examiner asserts that the '137 patent discloses the use of 7 α-dihydroequilenin in a method of reducing the risk of Alzheimer's disease and other dementia related conditions in humans.

It is well established that to anticipate under 35 U.S.C. §102, each and every element of a claimed invention must be disclosed in a single reference. *Apple Computer, Inc. v. Articulate Systems, Inc.*, 234 F.3d 14, 57 USPQ2d 1057 (Fed. Cir. 2000; *Brown v. 3M*, 265 F.3d 1349, 60 USPQ2d 1375 (Fed. Cir. 2001). Claim 20, as amended, is directed to methods for delaying or reducing the likelihood of, or ameliorating, a disease or disorder associated with amyloidosis, which method comprises administering an Aβ level reducing dose of ***17β-estradiol*** to a subject who has an increased risk for developing or shows a symptom of the disease or disorder associated with amyloidosis, wherein the dose of 17β-estradiol does not affect soluble APP levels.

Neurology 1999;52:965-970) that reported no relation of AD and HRT and an inverse relation of AD with lifetime HRT use, respectively (see page 2123, col. 1 of Zandi et al.).

The '137 patent fails to expressly or inherently disclose each and every element of claims 20, 21 and 23-25. The '137 patent discloses the use of 17 α-dihydroequilenin, a component of Premarin™ (conjugated equine estrogens), for the prevention of neurodegeneration and cognitive dysfunction associated with AD and other dementia-related disorders.

The '137 patent does not teach or suggest administering 17β-estradiol to a subject to delay, or reduce the likelihood of, or ameliorate a disease or disorder associated with amyloidosis, as required by claim 20. 17 α-dihydroequilenin and 17β-estradiol have different structures, which result in different levels of activity at the α- and β-estrogen receptors, and stimulation of different binding proteins. For example, 17 α-dihydroequilenin is a B-ring estrogen not native to humans, and has a greater potency in stimulating such binding proteins as cortisol-binding globulin and thyroid-binding globulin.

Thus, since the '137 patent does not contemplate or suggest the use of 17β-estradiol, the '137 patent fails to anticipate claims 20, 21 and 23-25.[2] Reconsideration and withdrawal of the rejection of claims 20, 21 and 23-25 under 35 U.S.C. §102(b), is requested.

Claims 1-3, 5-6, 20, 21, 24, and 25 stand rejected as allegedly being anticipated by Xu et al. (Nature Medicine, vol 4, April, 1998, pp. 447-451). The Examiner contends that Xu et al. disclose that estrogen reduces neuronal generation of Aβ-amyloid peptides, in particular Aβ42, and thereby delays or prevents AD.

Applicants respectfully traverse the foregoing rejection and submit that Xu et al. fail to teach or suggest each and every element of the claimed invention. The present invention describes the production of an ovariectomized (ovx) animal model that provides for the evaluation of compounds for the modulation of Aβ formation. This animal model was used to establish that administration of an estrogen compound reduces Aβ levels, and ***surprisingly does not affect soluble***

[2] Further, based on the differences between 17β-estradiol and 17 α-dihydroequilenin, one of skill in the art would not be motivated to modify the teachings of the '137 patent and replace 17 α-dihydroequilenin with 17β-estradiol. Thus, the pending claims are not obvious in view of the '137 patent.

APP levels in vivo. This was the first evidence that estrogen has an effect on Aβ levels in living animals.

Claim 1 is directed to methods for reducing a level of amyloid-β (Aβ) peptides ***in vivo***, comprising administering an Aβ level reducing dose of an estrogen compound ***to an animal having*** an increased level of Aβ, and where the dose of the estrogen compound does not affect soluble APP levels.

Claim 20 is directed to methods for delaying or reducing the likelihood of, or ameliorating, a disease or disorder associated with amyloidosis, which method comprises administering an Aβ level reducing dose of 17β-estradiol to a subject who has an increased risk for developing or shows a symptom of the disease or disorder associated with amyloidosis, where the dose of 17β-estradiol does not affect soluble APP levels.

Claims 1 and 20 are directed to ***in vivo methods***. *In vitro* results, such as those described in Xu et al., are by no means predictive of the *in vivo* effects of administration of estrogen compounds as described and claimed in the instant application, but even if they were, the results in Xu et al. do not teach or even suggest the claimed invention. Xu et al. do not describe any animal models whatsoever, thus this reference does not describe ***in vivo*** administration of an estrogen compound ***to an animal having an increased level of Aβ***, to reduce levels of Aβ, as required by claim 1. With respect to claim 20, Xu et al. fails to teach or suggest the administration of 17β-estradiol ***to a subject*** to delay, reduce the likelihood of, or ameliorate a disease or disorder associated with amyloidosis, where the subject has an increased risk for developing or shows a symptom of the disease or disorder associated with amyloidosis.

Xu et al. describes only ***in vitro*** studies to investigate the responses of soluble beta APP and Aβ to 17β-estradiol as a function of duration of hormone treatment (page 447, left col.). In these studies, N2a cells in tissue culture co-expressing human βAPP and the familial AD-linked PS1 variant, which generates readily detectable levels of Aβ, were maintained in the presence of a high dose of 17β-estradiol. As pointed out in the Applicants' previous Response (dated March 14, 2005), Xu et al. fail to teach or suggest that estrogen can be administered in doses which ***do not effect soluble APP*** levels yet which are effective in reducing levels of Aβ peptides. In fact,

according to Xu et al., treatment with 17 βE2 caused an ***increase*** in soluble APP release in the cells (see page 449, first paragraph of Xu et al.). Thus, the *in vitro* results described in Xu et al. cannot be used to predict *in vivo* effects of administration of estrogen compounds as claimed in the instant application.

Therefore, based on the foregoing, Xu et al. does not and can not anticipate the invention of claim 20. Accordingly, reconsideration and withdrawal of the foregoing rejection under 35 U.S.C. §102(b), is requested.

Rejections under 35 U.S.C. §103(a)

Claim 22 stands rejected as allegedly obvious over the '137 patent. The Examiner contends that all that is lacking in the '137 patent is administration of the estrogen compound for at least 10 days, and that it would have been obvious to one of ordinary skill in the art to administer the compound for at least 10 days.

Applicants respectfully traverse the foregoing rejection. As set forth above, the '137 patent does not teach or suggest methods for delaying or reducing the likelihood of, or ameliorating, a disease or disorder associated with amyloidosis, which method comprises administering an Aβ level reducing dose of 17β-estradiol to a subject who has an increased risk for developing or shows a symptom of the disease or disorder associated with amyloidosis, where the dose of 17β-estradiol does not affect soluble APP levels.

As set forth above, one of ordinary skill in the art would not be motivated to alter the teachings of the '137 patent and administer 17β-estradiol in place of 17 α-dihydroequilenin, as directed by the '137 patent. Thus, the '137 patent fails to teach or suggest each and every element of independent claim 20, upon which claim 22 depends, and therefore does not suggest, much less anticipate, the claimed invention. See M.P.E.P. §2143. Accordingly, Applicants respectfully request reconsideration and withdrawal of the foregoing rejection of claim 22 under 35 U.S.C. §103(a).

Claims 4, 22, 23 and 31-33 stand rejected as allegedly obvious over Xu et al. The Examiner asserts that Xu et al. lack "the use of estrogens other than estradiol, the use of estrogens in a controlled release device, and the amounts and protocol of administration." The Examiner contends that it would have been obvious to use conjugated estrogen with a reasonable expectation of success since estrogen receptors are the same and the conjugated estrogen is used in the art in estrogen replacement therapy. The Examiner asserts that the use of a controlled release device would have been obvious since these are commercially available and that administration for 10 days and the specific amounts are a "manipulatable parameter." The Examiner further contends that Xu et al. is "suggestive" of delaying or preventing AD and "the mechanism by which the same claimed compound taught by the prior art works has no significance." (Office Action, page 7).

Applicants respectfully traverse the foregoing rejection. As set forth above, Xu et al. fail to teach or suggest that estrogen can be administered in doses which do not effect soluble APP levels yet are effective in reducing levels of Aβ peptides. Xu et al. teach that treatment with 17β-estradiol increases soluble βAPP levels, which is contrary to the surprising discovery of the instant invention that the level of Aβ peptides are reduced ***in vivo*** without soluble APP levels being affected. Xu et al. describe only *in vitro* studies. Therefore, Xu et al. do not teach or suggest the present invention comprising administering an estrogen compound, *e.g.*, 17β-estradiol, ***to a subject***. Thus, Xu et al. fail to teach or suggest each and every element of the pending claims, and therefore does not suggest, much less anticipate, the claimed invention. See M.P.E.P. §2143. Accordingly, Applicants respectfully request reconsideration and withdrawal of the foregoing rejection of claims 4, 22, 23 and 31-33 under 35 U.S.C. §103(a).

Claims 1-6, 20-25 and 31-33 stand rejected as allegedly obvious over WO 99/48488 (herein after "the '488 publication") in combination with Washburn (U.S. Patent No. 5,510,342, hereinafter "the '342 patent"), Holland (U.S. Patent No. 3,843,662, hereinafter "the '622 patent") and Lundeen (Endocrinology, vol. 138, pp. 1552, 1997). The Examiner contends that the '488 publication teaches that blood cholesterol levels correlate with the production of amyloid protein and are predictors of populations at risk of developing AD. The Examiner also contends that the

'488 publication teaches methods of lowering cholesterol, which can be used to decrease production of Aβ, thereby decreasing the risk of developing AD. The Examiner acknowledges that the '488 publication lacks the use of estrogens and asserts that it would have been obvious to one of ordinary skill in the art to use estrogens for lowering the levels of Aβ peptide and decreasing the risk of AD because the secondary references teach that estrogens and conjugated estrogens lower cholesterol and because the '488 publication teaches that methods of lowering cholesterol can be used to decrease production of Aβ.

The Examiner further contends that the '488 publication clearly establishes the correlation between cholesterol levels, amyloid proteins and Alzheimer's disease and shows the effect of cholesterol lowering compounds in lowering the production of A beta thereby decreasing the risk of developing AD.

Applicants respectfully traverse the foregoing rejection. The '488 publication does not teach each and every limitation of the claimed invention. The '488 publication discloses that cholesterol lowering drugs can reduce production of Aβ. The secondary references (the '342 patent, the '622 patent and Lundeen) disclose that certain estrogens have cholesterol-lowering effects. However, none of these secondary references teach or suggest administering an estrogen compound in a dosage which is effective in reducing Aβ levels and which also does not affect soluble APP levels. The reduction of Aβ without the modulation of soluble APP levels *in vivo* after administration of an estrogen compound was a surprising and unexpected result of the experiments carried out by Applicants using ovx animal models as described in the specification. Thus, none of the cited references, either alone or in combination, teaches or suggests the claimed invention.

Furthermore, it has recently been shown that cholesterol is not correlated with Aβ formation. In particular, Fagan *et al.* ((2004) *American Journal of Pathology* Vol. 165, No. 4:1413; copy attached as Exhibit 2) crossed PDAPP mice, which are transgenic mouse models of AD-like cerebral amyloidosis, with apolipoprotein AI-null mice with reduced plasma cholesterol levels. It was shown that there were "no differences in the Aβ pathology in PDAPP mice of various apoAI genotypes despite robust differences in plasma cholesterol levels between the groups" (see Fagan *et al.*, Abstract). In particular, Fagan *et al.* show that levels of apolipoprotein E (ApoE), not

cholesterol *per se*, play a primary role in brain Aβ metabolism. Thus, based on the finding that cholesterol levels are not directly related to Aβ pathology, one of ordinary skill in the art would not be motivated to use estrogen to reduce cholesterol and thereby reduce production of Aβ.

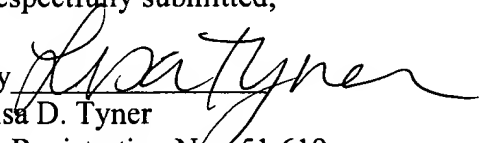
Reconsideration and withdrawal of the obviousness rejection is respectfully requested.

Conclusion

Applicant respectfully requests entry of the foregoing amendments, remarks, and evidence in the file history of this application. In view of the above, Applicant believes the pending claims are in condition for allowance and earnestly solicits allowance of the claims.

Dated: December 9, 2005

Respectfully submitted,

By 

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ORIGINAL CONTRIBUTION

Hormone Replacement Therapy and Incidence of Alzheimer Disease in Older Women

The Cache County Study

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Context Previous studies have shown a sex-specific increased risk of Alzheimer disease (AD) in women older than 80 years. Basic neuroscience findings suggest that hormone replacement therapy (HRT) could reduce a woman's risk of AD. Epidemiologic findings on AD and HRT are mixed.

Objective To examine the relationship between use of HRT and risk of AD among elderly women.

Design, Setting, and Participants Prospective study of incident dementia among 1357 men (mean age, 73.2 years) and 1889 women (mean age, 74.5 years) residing in a single county in Utah. Participants were first assessed in 1995-1997, with follow-up conducted in 1998-2000. History of women's current and former use of HRT, as well as of calcium and multivitamin supplements, was ascertained at the initial contact.

Main Outcome Measure Diagnosis of incident AD.

Results Thirty-five men (2.6%) and 88 women (4.7%) developed AD between the initial interview and time of the follow-up (3 years). Incidence among women increased after age 80 years and exceeded the risk among men of similar age (adjusted hazard ratio [HR], 2.11; 95% confidence interval [CI], 1.22-3.86). Women who used HRT had a reduced risk of AD (26 cases among 1066 women) compared with non-HRT users (58 cases among 800 women) (adjusted HR, 0.59; 95% CI, 0.36-0.96). Risk varied with duration of HRT use, so that a woman's sex-specific increase in risk disappeared entirely with more than 10 years of treatment (7 cases among 427 women). Adjusted HRs were 0.41 (95% CI, 0.17-0.86) for HRT users compared with nonusers and 0.77 (95% CI, 0.31-1.67) compared with men. No similar effect was seen with calcium or multivitamin use. Almost all of the HRT-related reduction in incidence reflected former use of HRT (9 cases among 490 women; adjusted HR, 0.33 [95% CI, 0.15-0.65]). There was no effect with current HRT use (17 cases among 576 women; adjusted HR, 1.08 [95% CI, 0.59-1.91]) unless duration of treatment exceeded 10 years (6 cases among 344 women; adjusted HR, 0.55 [95% CI, 0.21-1.23]).

Conclusions Prior HRT use is associated with reduced risk of AD, but there is no apparent benefit with current HRT use unless such use has exceeded 10 years.

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COMPARED WITH MEN, WOMEN appear to be at increased risk of Alzheimer disease (AD) after ages 80 to 85 years.[1-3] Postmenopausal depletion of endogenous estrogens may contribute to this risk. Estrogens may exert several neuroprotective effects on the aging brain, including inhibition of β-amyloid formation, stimulation of cholinergic activity, reduction of oxidative stress-related cell damage, and protection against vascular risks.[4]

Several studies have examined whether hormone replacement therapy (HRT) is associated with reduced risk of AD in older women. Early case-control study results of this association were mixed.[5-13] One such study reported no relation of AD and HRT ascertained from pharmacy records within a 10-year period of observation.[14] Another study using prescription records showed an inverse relation of AD with lifetime HRT use.[15] Two prospective studies[16,17] suggested a benefit of lifetime HRT use, but the most recent study,[18] conducted using the UK General Practice Research Database, showed no relation of AD to HRT prescriptions within a 10-year period of ob-

For editorial comment see p 2170.

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servation. Thus, the relationship of HRT and AD remains uncertain.

In the large Cache County cohort,[3] we analyzed prospective data on the association of HRT and AD in elderly women. We examined whether a reduction in risk with HRT, if any, varied with the number of ε4 alleles at *APOE*, the polymorphic genetic locus for apolipoprotein E. Finally, we assessed whether apparent benefits with HRT varied in relation to duration and recency of exposure.

METHODS

Study Population

The Cache County Study is a longitudinal investigation of the prevalence and incidence of AD and other dementias in relation to genetic and environmental risk factors. Details of the study protocol have been published previously.[3,19] Briefly, between 1995-1997 we used a multistage screening and assessment protocol (wave I) to diagnose cases of dementia among 5677 elderly residents of Cache County, Utah. More than 97% of the 5092 initial participants (90% of those aged ≥65 years, including 2928 women) provided buccal DNA for genotyping at *APOE*. Three years later, between 1998-2000, we used similar procedures to diagnose new cases of dementia (wave II) among the surviving at-risk population of 4119 (2401 women).[3] Essentials of the screening procedures and study protocol are shown in FIGURE 1.

Participants were screened with the Modified Mini-Mental State examination (3MS)[20] or, for those unable to participate, an informant questionnarie[21] followed by the Dementia Questionnaire (DQ)[22] administered to collateral informants (spouses, companions, or others knowledgeable about the respondents). Participants with screening results suggesting a cognitive disturbance then underwent a clinical assessment. Collateral informants provided a medical history, a dementia symptom checklist, and a chronological history of cognitive symptoms; specially trained nurses conducted a structured neurological examination; and psychometric technicians administered a 1-hour battery of neuropsychological tests. A geriatric psychiatrist and neuropsychologist then reviewed the results and assigned working diagnoses of dementia (*Diagnostic and Statistical Manual of Mental Disorders, Revised Third Edition* criteria) or other cognitive syndromes; 83.9% of these subjects still living were then examined by a board-certified geriatric psychiatrist, and among these, 65.9% underwent routine laboratory diagnostic testing for differential diagnosis. All this information was then considered by a panel of experts, who identified dementia and assigned diagnoses of AD[23] and other disorders using standard criteria.

Among all study participants, we identified 152 individuals (98 women, 54 men) with incident dementia. To these we added 33 individuals (25 women, 8 men) who had an onset of dementia detected in the later stages of wave I (before the start of wave II), yielding a total of 185 incident cases (123 women, 62 men). The estimated sensitivity of the screening protocol for detection of incident dementia was 89% (K. Hayden et al, unpublished data, 2002). Of the women with incident dementia, 88 had diagnoses of definite, probable, or possible AD.[23] A second diagnosis of another dementing illness was entered for 12 of these AD cases. Of the 62 men with incident dementia, 35 had an AD diagnosis, 8 of these with another dementing illness. A comparison with neuropathological findings in 54 individuals suggested that the accuracy of our AD diagnoses is similar to typical rates reported from university AD clinics (eg, positive predictive value, 90%; B. Plassman et al, unpublished data, 2002). Another 1801 women completed the wave II study procedures sufficiently to assess their cognitive status and were found to be free of dementia. Of these, 298 underwent all stages of evaluation, including clinical assessment; the other 1503 showed no evidence of dementia on screening measures and were not further evaluated. Unaffected men numbered 1322, of whom 249 completed a clinical assessment.

Exposure Assessment

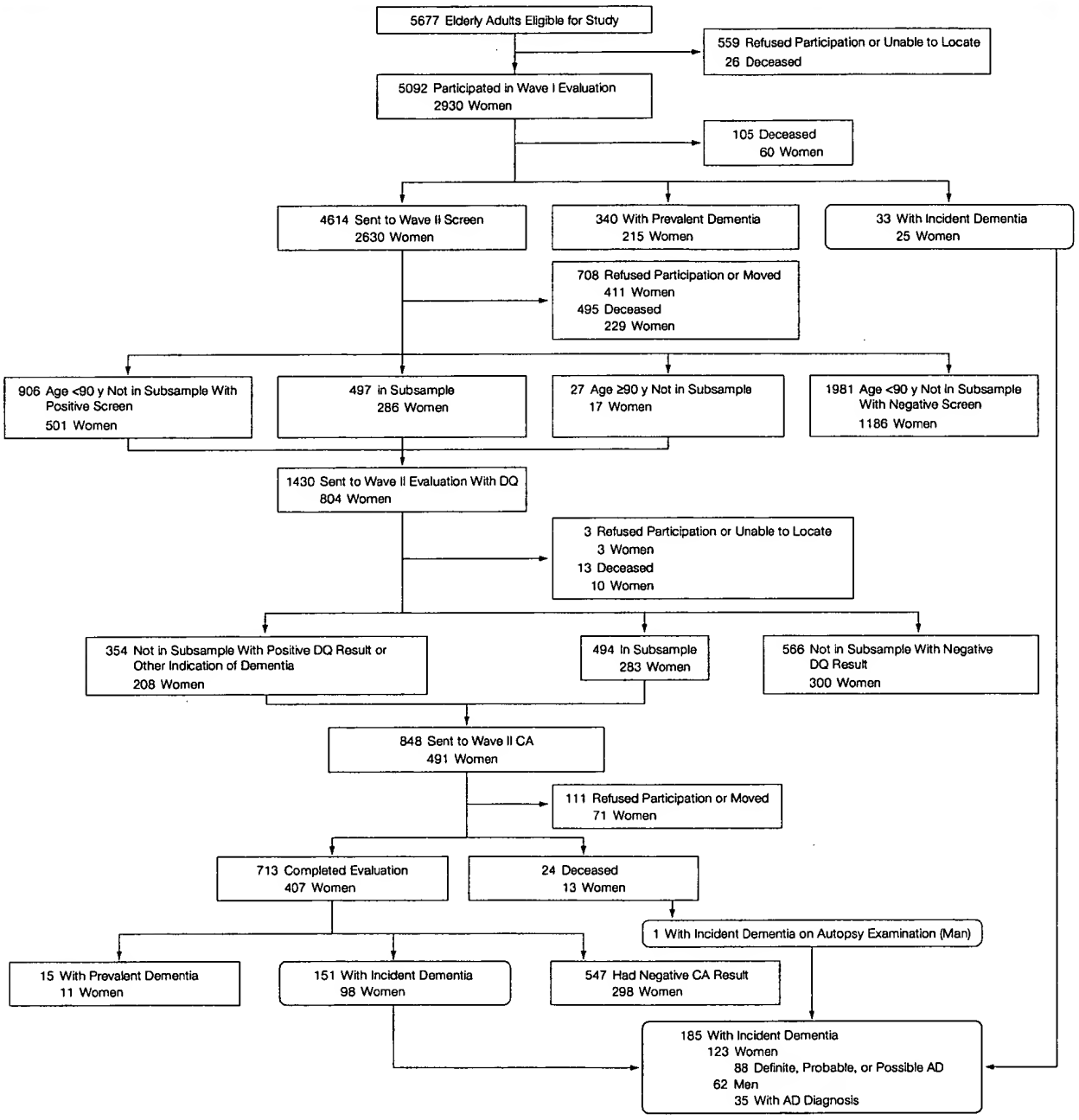
The initial wave I interview provided 2 sources of information on HRT. Women were asked if they had ever taken HRT and, if so, for how long. They were also asked about use over the prior 2 weeks of any medicines, including HRT. Interviewers then viewed these current medications and recorded the name, dose, and usage indication for each. Although 18 women developed incident AD within 30 months of their wave I interviews, none appeared to have substantial cognitive impairment when interviewed, and all therefore provided their own exposure information.

We first classified HRT according to report of lifetime use, categorizing participants as "exposed" if they endorsed ever having taken HRT or if HRT was among their current medicines. Complete data for HRT exposure were available from 1866 (98.8%) of the 1889 women. Omitting 10 HRT users (1%) who did not report their duration of use, we classified exposures into duration strata of less than 3 years, 3 to 10 years, and more than 10 years. Finally, we classified exposed women as current vs former users, the latter being individuals who endorsed HRT exposure at some point but did not have HRT among their current medicines. Among the current HRT users, 72% were taking an unopposed oral estrogen preparation.

Statistical Analysis

We compared characteristics of HRT users and nonusers using χ^2 tests for categorical variables and 2-sample *t* tests for continuous measures. We then used discrete-time survival analysis[24] to compare risks of incident AD among HRT users and reference groups of nonusers and of men. We considered each year under observation as a discrete time interval. Participants entered the analytic pool at the age of their wave I interview and were then considered year by year until they either developed AD or underwent wave II screening. Hazard ratios (HRs) were estimated by odds ratios in logistic models that accommodated multiple covariates.

Figure 1. Screening Procedures and Protocol for Detection and Evaluation of Dementia and Alzheimer Disease in the Cache County Study



An at-risk population of 4614 was identified for screening at wave II. These included a high-risk subsample of 497 participants identified previously (wave I)[19] who were asked to complete all phases of the protocol regardless of their screening results. Based on results of screening with the Modified Mini-Mental State examination (3MS)[20] or, for those unable to participate, the Informant Questionnaire for Cognitive Disorders in the Elderly (IQCODE)[21] and the Dementia Questionnaire (DQ)[22] administered to collateral informants of selected participants, we sought a clinical assessment (CA) of 848 individuals. Among 713 completed CAs, we identified 151 individuals with incident dementia and 15 whose (prevalent) dementia had gone undetected in wave I. One individual died before we could complete the examination, but a brain autopsy confirmed the presence of Alzheimer disease (AD). Thirty-three individuals with milder cognitive syndromes developed incident dementia during the later stages of wave I. The 185 individuals with incident dementia included 123 with AD (88 women). A comparison group of 3123 unaffected participants (1801 women) included the following: 1981 participants (1186 women) who were not in the subsample but screened negative on the 3MS/IQCODE; 566 participants (300 women) who were not in the subsample but screened negative on the DQ; 547 participants (298 women) who underwent CA and were found to be free of dementia; and 29 participants (17 women) in the subsample who refused to participate, died, or moved away prior to the CA but showed no evidence of cognitive disturbance in their screening results.

Table 1. Demographic Characteristics by Hormone Replacement Therapy (HRT) Use of the Men and Women Completing Waves I and II of the Cache County Study (n = 3246)

	Men	Women With No HRT Use	Women With Any HRT Use	Women Missing HRT Use Data
No.	1357	800	1066	23
Age, mean (SD), y	73.2 (6.1)*	76.2 (7.0)	73.1 (5.8)†	79.1 (8.7)
Years of education, mean (SD)	14.1 (3.4)*	12.7 (2.3)	13.1 (2.2)†	12.5 (3.5)
APOE ϵ4 alleles, No. (%)				
0	930 (68.5)	549 (68.6)	735 (68.9)	13 (56.5)
1	378 (27.9)	224 (28.0)	305 (28.6)	7 (30.4)
2	40 (2.9)	17 (2.1)	23 (2.2)	0 (0)
Missing	9 (0.7)	10 (1.3)	3 (0.3)	3 (13.0)
Alzheimer disease, No. (%)				
Yes	35 (2.6)*	58 (7.3)	26 (2.4)†	4 (17.4)
No	1322 (97.4)*	742 (92.8)	1040 (97.6)†	19 (82.6)

*Difference compared with all women significant at $P<.01$.
†Difference compared with HRT nonusers significant at $P<.01$.

We fit a series of models that were built on a "base model" for AD incidence that had previously yielded a good fit to the data for both men and women.[3] That model included terms for age, age-squared, and years of education, as well as dummy-coded terms for the presence of 1 or 2 *APOE* ϵ4 alleles, and interactions between age and the *APOE* ϵ4 terms. It also included terms for sex and its statistical interaction with age, but the current analyses that considered only women omitted those terms. We fit the discrete-time logistic models using SAS version 8 software (SAS Institute Inc, Cary, NC) and report parameter estimates with 95% profile likelihood confidence intervals (CIs).

RESULTS

TABLE 1 presents the characteristics of the current analytic sample of men and women, the latter categorized by HRT use. Missing data on HRT use were relatively rare; women who did not provide this information tended to be older and slightly less educated than women who did. There were 411 living women who did not participate in the initial assessment of wave II; they were less likely to report HRT use ($P<.001$) and had lower 3MS scores ($P<.001$) at baseline than participating women. Among the remainder, 1066 women (56.4%) reported use of HRT at any time, with a mean exposure duration of 11.6 years. These users were significantly younger and more educated than nonusers.

Between the initial interview and the follow-up procedures (3 years), 35 men (2.6%) and 88 women (4.7%) developed AD. Univariate analyses suggested that AD was significantly more common for women than for men ($\chi^2_1=9.37$, $P=.002$), but less common among women with a history of HRT compared with nonusers ($\chi^2_1=24.62$, $P<.001$). Similarly, unadjusted estimates of the hazard for AD (TABLE 2, models 1 and 2) were significantly higher for women than for men, but were lower among women who reported HRT use than a reference group of nonusers.

We next constructed a series of multiple discrete-time logistic models that included the covariates of the base model[3] (FIGURE 2A and the remainder of Tables 2 and 3). Figure 2A shows AD incidence modeled for men and women with 13 years of education (the sample median) and no ϵ4 alleles at *APOE* (the most numerous group). The hazard for men and for women appears roughly equal until age 80 years, but the base model's significant sex-by-age interaction term[3] implies a substantial added risk for women after this age. This risk is indicated by an adjusted HR of 2.11 (95% CI, 1.22-3.86) among women vs men older than 80 years.

We next estimated the modification in women's risk with HRT after controlling for the covariate terms of the base model (Table 2, model 3). Comparing this adjusted estimate with the unadjusted figure (model 2) showed only a slight shift of the HR toward the null. The adjusted estimate did not change appreciably (results not shown) when we added terms separately for comorbid conditions including diabetes mellitus, cardiovascular disease, and depression, as well as for the use of nonsteroidal anti-inflammatory drugs (NSAIDs).[25] To investigate whether the apparent reduction in AD risk among HRT users might simply reflect their tendency toward a healthy lifestyle, we also added terms post hoc for use of multivitamins and of calcium supplements (both obtained at the initial wave I interview) as plausible indicators of such a tendency. Model 4 shows that neither of these terms was significantly associated with risk of AD. Their inclusion as covariates also yielded no appreciable change in the point estimate of the relative hazard among HRT users. To examine whether the HRT effect varied with age or with number of *APOE* ϵ4 alleles, we added terms to model 3 for interactions between HRT and these covariates. Lack of an apparent interaction between HRT and age (model 5) suggested that the effect with HRT did not vary over the life span. The interactions between HRT and presence of 1 or 2 *APOE* ϵ4 alleles also failed to reach statistical significance (model 6), although there was some suggestion that risk reduction with HRT may be greater in women with 2 ϵ4 alleles ($P=.19$).

TABLE 3 shows variation in the apparent HRT effect with duration and recency of exposure. We first examined risk estimates among the 3 categories of usage duration (model 7). Longer duration was associated with greater reduction in risk of AD. Figure 2B shows this graphically, depicting the age-specific hazards modeled for women with no *APOE* ϵ4 alleles and 13 years of education; Figure 2B also shows the risk for men with these same characteristics. The increased hazard of AD among women vs men in late old age is again apparent. The added risk for women appears greatest for those with no reported use of HRT.

This sex-specific risk was attenuated, however, with increasing years of HRT exposure. The estimated hazard for women who had used HRT for more than 10 years was similar to that for men (vs men, adjusted HR, 0.77; 95% CI, 0.31-1.67).

Model 8 shows risk estimates for women with HRT use after separation of current and former users. Compared with nonusers, only former users showed significantly reduced risk. Partitioning as before into 3 categories of usage duration (model 9), we observed an incremental reduction in apparent risk for former users with longer history of use. Former users with more than 10 years of exposure had an estimated 5-fold lower risk of AD. Among current users, however, there was no suggestion of reduced risk with 10 or fewer years of exposure, and only a modest reduction thereafter among 344 women.

COMMENT

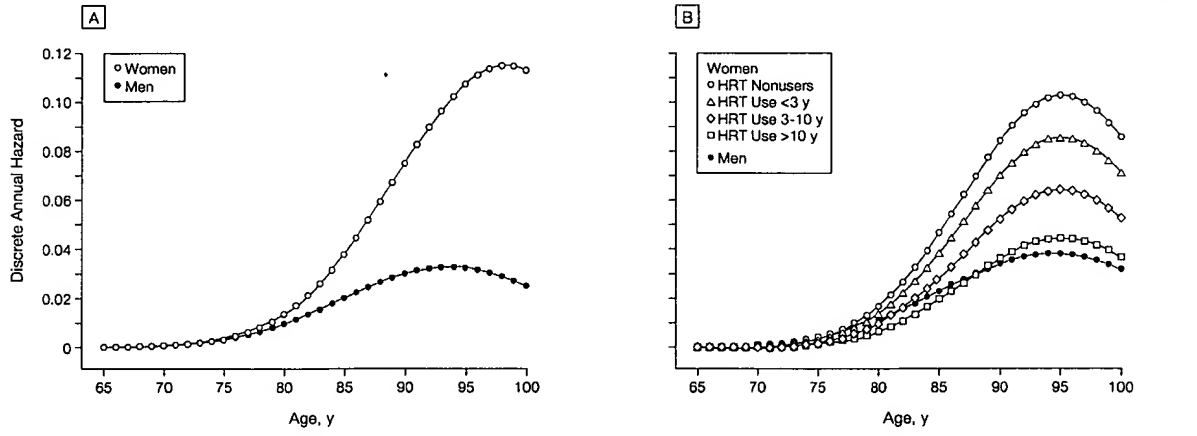
These findings extend those of 2 previous prospective studies[16,17] and provide new evidence to suggest a protective effect of HRT. As in the previous studies, the adjusted risk of incident AD among lifetime HRT users was reduced to little more than half that among nonusers. This effect appeared to be stronger among women with 2 ϵ4 alleles at *APOE*, but given the small numbers available, the interpretation of this finding is uncertain. One previous prospective study examined the effects with HRT across *APOE* genotypes, suggesting a slightly greater apparent effect with HRT in women who had 1 ϵ4 allele.[16] Only half the sample in that study had been genotyped at *APOE*, however, and none of the 9 women with 2 ϵ4 alleles in that study had ever used HRT.

Table 2. Relative Hazards for Alzheimer Disease in Women Estimated From Discrete-Time Logistic Regression Models

	Relative Hazard (95% Confidence Interval)					
Terms*	Model 1†	Model 2†	Model 3	Model 4	Model 5‡	Model 6
Female sex	1.82 (1.24-2.73)	. . .	. . .	. . .	. . .	. . .
Any HRT		0.33 (0.21-0.52)	0.59 (0.36-0.96)	0.63 (0.38-1.02)	0.49 (0.22-1.04)	0.66 (0.34-1.23)
Calcium supplements				0.85 (0.52-1.39)	. . .	. . .
Multivitamins				1.08 (0.62-1.83)	. . .	. . .
HRT by age					1.03 (0.94-1.12)	. . .
HRT by 1 *APOE* ϵ4 allele						1.01 (0.36-2.81)
HRT by 2 *APOE* ϵ4 alleles						0.25 (0.01-1.85)

*Reference group for each term shown includes women nonusers of the compound represented by that term, except for sex where the reference group is men. HRT indicates hormone replacement therapy.
†Models 1 and 2 are simple unadjusted bivariate models; models 3-6 are built on a "base model" that includes terms (not shown) for age, age-squared, years of education, dummy-coded terms for the presence of 1 or 2 *APOE* ϵ4 alleles, and interactions between age and the dummy-coded *APOE* terms.
‡The HRT term in model 5 with the HRT-by-age interaction was estimated at the mean age of 76 years.

Figure 2. Estimated Discrete Annual Hazard of Alzheimer Disease for Men and Women by Age, and by Duration of Hormone Replacement Therapy Use for Women



Both figures indicate risks estimated for an individual with the mean value of 13 years of education and no ϵ4 alleles at *APOE*. A, The curves depict the annual hazards predicted by fitting the base model including an age-by-sex interaction term. The annual hazard for Alzheimer disease (AD) appears similar for men and women before 80 years of age but diverges rapidly afterward with an excess risk found in women. B, The curves depict the annual hazards predicted by fitting model 7 of Table 3 to the women with available hormone replacement therapy (HRT) exposure information and, in filled circles, the corresponding annual hazards for men after omitting the terms for HRT. There were 35 instances of incident AD among 1357 men. Ordinate values for women differ slightly from those in panel A due to omission of women lacking HRT exposure information, several of whom experienced incident dementia.

Table 3. Relative Hazards of Alzheimer Disease (AD) in Women With Different Degrees of Duration and Recency of Hormone Replacement Therapy (HRT) Use, as Estimated From Discrete Time Logistic Regression Models

Terms*	Total No. (No. With AD)	Age, Mean (SD), y	Relative Hazard (95% Confidence Interval) Model 7†	Model 8†	Model 9†
HRT use, y					
<3	310 (10)	73.6 (5.8)	0.82 (0.38-1.57)	...	...
3-10	319 (8)	72.3 (5.8)	0.60 (0.26-1.22)	...	...
>10	427 (7)	72.8 (5.7)	0.41 (0.17-0.86)	...	...
HRT use					
Former	490 (9)	74.5 (5.9)		0.33 (0.15-0.65)	...
Current	576 (17)	71.9 (5.4)		1.08 (0.59-1.91)	...
HRT use, y					
Former					
<3	252 (6)	73.8 (5.7)			0.58 (0.22-1.27)
3-10	146 (1)	74.9 (6.0)			0.32 (0.08-0.68)
>10	83 (1)	75.4 (6.3)			0.17 (0.01-0.80)
Current					
<3	58 (4)	73.0 (6.2)			2.41 (0.70-6.34)
3-10	173 (7)	70.9 (5.0)			2.12 (0.83-4.71)
>10	344 (6)	72.1 (5.3)			0.55 (0.21-1.23)

*All models are built on a "base model" that includes terms (not shown) for age, age-squared, years of education, dummy-coded terms for the presence of 1 or 2 *APOE* ε4 alleles, and interactions between age and the dummy-coded *APOE* terms.
†Reference group for each analysis is nonusers of HRT.

We observed a distinct relation between AD risk and duration of HRT use. Two previous studies reported a similar result on dichotomizing duration at 1 year of use.[10,16] We observed considerably stronger effects with longer duration of usage. Compared with nonusers, Cache County women who had used HRT for more than 10 years experienced 2.5-fold lower incidence, comparable with the risk observed in men. Others have speculated that the lower rates in older men may reflect their greater availability of circulating testosterone, which may be converted in the central nervous system by aromatase to estradiol.[26] Taken to their logical conclusion, our findings suggest that if women were to use long-term postmenopausal HRT, their excess risk of AD over that of men in late old age might disappear.

A new finding in this study is an apparent limited window of time during which sustained HRT exposure seems to reduce the risk of AD. We found that, in contrast with earlier use, HRT exposures within 10 years of AD onset yielded little, if any, apparent benefit. These results are in accord with prior findings of reduced cognitive decline in elderly women who initiated HRT at menopause, but not in those with more recent exposures.[27] In fact, our results and those of all prior observational studies are consonant with a loss of HRT effect from exposures near the onset of dementia. A similar finding was reported recently for NSAIDs.[28] The results with both HRT and NSAIDs suggest that potentially neuroprotective agents may be useful only in the latent pathogenetic stages of AD, before there is extensive damage to the integrity of the brain. Limitation of the benefit of HRT to the latent stages of AD is also consistent with recent randomized treatment trials that suggest HRT is not effective in mitigating the progression of cognitive decline in women with established AD.[29-31]

Some have suggested that HRT may be most beneficial at menopause, when a precipitous depletion of endogenous estrogens may have greatest deleterious effect on neurons.[30] We were unable to test this hypothesis directly, but our findings are consistent with it: many women who had used HRT for more than 10 years before our wave I interview would likely have been exposed many years prior to the time when they became vulnerable to the onset of dementia. Furthermore, we found a reduced risk with HRT among former users but not among current users unless the latter had used HRT for more than 10 years. This last observation may explain the contrast in the findings of the 2 prior prospective studies[16,17] with those of 2 well-designed case-control studies that evaluated the relation of AD onset to prescription records within a 10-year interval.[14,18]

Our study capitalized on several characteristics of the Cache County population. Its residents are well educated and relatively homogeneous in their sociodemographic characteristics, including their tendency toward healthy lifestyles. They offer high response rates in research, and they enjoy remarkably long lives. Consequently, the current study may be less susceptible than some to response or healthy user biases. Further, we attempted to control for the latter bias in our analyses by testing a model with terms for multivitamin and calcium supplement use. Only those who took HRT showed a significantly reduced risk of AD.

Among potential limitations, the unusual sociocultural attributes of the Cache County sample may suggest a lack of generalizability of our findings to other populations, although this is less worrisome with biological measures than with social or cultural ones. Another potential limitation is that we observed a relatively short period of follow-up between wave I and wave II.

A common difficulty in pharmacoepidemiologic studies is incomplete recall of drug exposures. Faulty recall that is not related to the later occurrence of incident AD (*nondifferential exposure misclassification*) would reduce the observed strength of any real association between HRT and incident AD. Of greater concern is biased recall, in which exposures are underreported by women who are destined subsequently to develop AD (*differential exposure misclassification*). This form of bias may be of particular concern for the 18 women whose AD was detected in the later stages of wave I. However, the threat of incomplete recall should be lower with HRT than with most other medicines, because the use of HRT after menopause is a major life

decision for most women, almost always made in consultation with a physician. Furthermore, because HRT is typically used for several years, it is less likely to be forgotten than other, more transient drug exposures. Also, regarding possible differential or biased recall, we found no relationship between AD incidence and recollection of several other control exposures, including calcium and multivitamin supplements. It seems unlikely that women who later develop dementia would selectively forget their carefully considered decision to use HRT, but would accurately recall their use of these other compounds.

An important limitation of this and all other observational studies is unsuspected confounding. We cannot exclude the possibility that HRT users differ from nonusers in other attributes related to health in general and to AD in particular. Specifically, we considered whether current HRT users of short duration might have initiated use because they were concerned about mild (possibly prodromal) memory difficulties and had learned of other recent evidence for possible neuroprotective benefits of HRT. We discount this possibility, however, because all current users were taking oral estrogen preparations, available only by prescription. Numerous conversations over several years with the county's physicians failed to reveal any practitioner prescribing HRT for this indication. Nonetheless, the only way definitively to avoid this sort of difficulty is to conduct large-scale randomized prevention trials. Two such trials are currently in progress.[30,32] Our observations suggest that the benefits of HRT, if any, may take years to appear, and a considerable latency period may intervene between treatment and perceptible effect. Thus, caution would be in order when interpreting null or disappointing early trial results. Our findings, along with other recent work, suggest that HRT may be effective for the primary prevention of AD—if not for its treatment—and that patience in awaiting definitive trial results is indicated.

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ApoAI Deficiency Results in Marked Reductions in Plasma Cholesterol But No Alterations in Amyloid-β Pathology in a Mouse Model of Alzheimer's Disease-Like Cerebral Amyloidosis

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Epidemiological studies suggest links between cholesterol metabolism and Alzheimer's disease (AD), with hypercholesterolemia associated with increased AD risk, and use of cholesterol-lowering drugs associated with decreased risk. Animal models using cholesterol-modifying dietary or pharmacological interventions demonstrate similar findings. Proposed mechanisms include effects of cholesterol on the metabolism of amyloid-β (Aβ), the protein that deposits in AD brain. To investigate the effect of genetic alterations in plasma cholesterol on Aβ pathology, we crossed the PDAPP transgenic mouse model of AD-like cerebral amyloidosis to apolipoprotein AI-null mice that have markedly reduced plasma cholesterol levels due to a virtual absence of high density lipoproteins, the primary lipoprotein in mice. Interestingly and in contrast to models using non-physiological high fat diets or cholesterol-lowering drugs to modify plasma cholesterol, we observed no differences in Aβ pathology in PDAPP mice of the various apoAI genotypes despite robust differences in plasma cholesterol levels between the groups. Absence of apoAI also resulted in reductions in brain but not cerebrospinal fluid cholesterol, but had no effect on brain apolipoprotein E levels. These and other data suggest that it is perhaps the level of brain apolipoprotein E, not cholesterol *per se*, that plays a primary role in brain Aβ metabolism. *(Am J Pathol 2004, 165:1413–1422)*

Recent evidence suggests a link between cholesterol metabolism and the pathogenesis of Alzheimer's disease (AD). Epidemiological studies report positive associations between hypercholesterolemia (high plasma cholesterol levels) and risk for AD,[1–4] although findings are inconsistent.[5] Potentially consistent with such a link is the observation that the ε4 allele of apolipoprotein E (apoE), the isoform associated with elevated levels of plasma cholesterol,[6] is also the strongest genetic risk factor for late-onset AD.[7] ApoE4 also influences the age of clinical disease onset in families exhibiting an AD-causing gene mutation[8] and in AD associated with Down syndrome.[9] Finally, retrospective epidemiological studies demonstrate associations between use of HMG-Co-A reductase inhibitors (the cholesterol-lowering drugs known as statins) and reduced AD prevalence[10] and dementia risk.[11]

Experimental studies suggest a potential mechanism by which cholesterol influences AD may be via effects on the metabolism of amyloid-β (Aβ), the protein that accumulates and deposits in the AD brain. Cholesterol is found in dense core plaques in AD and transgenic mouse models of AD-like cerebral amyloidosis.[12] In addition, a portion of Aβ in plasma and cerebrospinal fluid (CSF) is associated with cholesterol-containing lipoproteins[13–16] and thus may be influenced by processes governing

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lipoprotein metabolism. Cholesterol can regulate amyloid precursor protein (APP) processing and Aβ generation *in vitro*,[17–19] and alterations in Aβ deposition have been observed in animal models of hyper- and hypocholesterolemia induced by high fat diets[20–23] or treatment with cholesterol-lowering drugs,[19,24] respectively. Finally, data from recent clinical trials demonstrate decreases in serum Aβ[25] and APP metabolites in CSF[26] after statin treatment, although other studies report minimal effects.[27,28]

While these data are suggestive, several issues must be resolved. With the exception of one study,[23] experimental high fat diets can be considered non-physiological because of other pathological consequences, including vascular inflammation and blood-brain barrier disruption.[29] In addition, potential effects of cholesterol-lowering drugs on AD risk differ for the various compounds despite equivalent cholesterol-lowering capabilities.[10,11] The statins also have pleiotropic effects (including anti-inflammatory, vascular, and antioxidant effects)[30] apart from their ability to lower cholesterol, thus raising the question of mechanism of action. Therefore, to circumvent the limitations and caveats of previous studies, we used a direct genetic approach to investigate whether life-long, non-dietary, non-pharmacological differences in plasma cholesterol levels influence the development of Aβ-related pathology in a well-characterized transgenic mouse model of AD-like cerebral amyloidosis. Genetic variations in plasma cholesterol levels in APP^{V717F} (PDAPP) transgenic mice were achieved by modifying apoAI gene dose through breedings to $apoAI^{-/-}$ mice, known to exhibit marked deficiencies in plasma cholesterol level.[31,32] We observed significant reductions in plasma cholesterol in $PDAPP^{+/-}$, $apoAI^{-/-}$ mice, but no differences in brain Aβ pathology. Absence of apoAI also resulted in significant reductions in cholesterol measured in brain but had no effect on brain apolipoprotein E (apoE) levels. These data suggest that it is perhaps the level of brain apoE, and not cholesterol *per se*, that may be playing a primary role in brain Aβ metabolism.

Materials and Methods

Animals and Tissue Preparation

Transgenic mice expressing APP^{V717F} (PDAPP;[33] were bred with mice lacking the gene for apolipoprotein AI ($apoAI^{-/-}$)[31] (Jackson Labs, Bar Harbor, ME) to ultimately generate $PDAPP^{+/-}$ mice expressing two ($apoAI^{+/+}$), one ($apoAI^{+/-}$), or no ($apoAI^{-/-}$) copies of the endogenous mouse apoAI gene within the same litter. PDAPP animals were on a mixed (50% C57BL/6/DBA, 50% Swiss Webster) background,[34] and $apoAI^{-/-}$ mice were on a C57BL/6 background. Animals were screened for the presence of the APP^{V717F} transgene[35] and apoAI genes (Jackson Labs) by PCR from tail DNA. ApoAI genotype was further confirmed by semi-quantitative Western blotting of plasma (see below). Animals were sacrificed at 6, 9, 12, or 15 months of age. Mice were anesthetized with sodium pentobarbital, and CSF was collected from the cisterna magna as described,[36] and blood (for plasma) was obtained via cardiac puncture. Following transcardial perfusion with 0.1 mol/L phosphate-buffered saline (PBS) (pH 7.4), brains were divided into left and right hemispheres. The right hemisphere was immersion-fixed in paraformaldehyde (4% in 0.1 mol/L phosphate buffer, pH 7.4) overnight and cryoprotected for 24 hours in 30% sucrose in PBS at 4°C for subsequent histological analysis. The left hemisphere was regionally dissected and frozen in dry ice for subsequent biochemical analysis.

Histological Analysis

Tissue sections were cut at 50 μm in the coronal plane on a freezing sliding microtome from the genu of the corpus callosum through the caudal extent of the hippocampus. For analysis of Aβ-immunoreactive (IR) deposits, sections were immunostained with a pan anti-Aβ antibody (Biosource; Camarillo, CA) as described.[37] Thioflavine-S (Thio-S) staining was used to identify amyloid (ie, fibrillar Aβ), as described.[35] Quantitative analysis of Aβ and amyloid deposition in the hippocampus was performed, defined as the percent hippocampal area covered by Aβ-IR and Thio-S-positivity, respectively, in three tissue sections, 300 μm apart starting 900 μm caudal to the beginning of the hippocampus in coronal section. The percentage of hippocampal area covered by Aβ-IR or Thio-S-positivity (% Aβ or amyloid load, respectively) was determined in an unbiased fashion using the Cavalieri point counting method[38,39] with the assistance of a stereology system (MicroBrightField, Inc.; Colchester, VT). Statistical comparisons were made with analysis of variance followed by Tukey post-hoc tests using GraphPad Prism software (version 4.0) for Windows (San Diego, CA). In addition, sections from a subset of animals of each genotype displaying amyloid deposition at 15 months of age were stained with the de Olmos silver stain[40] to identify neuritic dystrophy associated with amyloid plaques. Power calculations indicate that we can detect a 30 to 40% difference in the amount of Aβ deposition between groups (at 15 months of age) using 10 to 15 animals per group.

Biochemical Analysis

Soluble and insoluble fractions of brain tissue were prepared for Aβ analysis as described.[41] Half of the hippocampus from each animal was Dounce homogenized in carbonate buffer (100 mmol/L Na_2CO_3, 50 mmol/L NaCl, pH 11.5) containing protease inhibitors (20 μg/ml aprotinin, 10 μg/ml leupeptin) and centrifuged at 14,000 rpm for 20 minutes at 4°C. The supernatant (soluble fraction) was transferred to another tube, kept on ice, and immediately analyzed (see below). The pellet was then homogenized in 5 mol/L guanidine buffer (5 mol/L guanidine-HCl in 50 mmol/L Tris-HCl, pH 8.0) and rotated for 3.5 hours at room temperature (RT). Following centrifugation at 14,000 rpm for 20 minutes at 4°C, the supernatant (insoluble fraction) was transferred to another tube

and stored at −70°C until analyzed. Levels of human $A\beta_{40}$ and $A\beta_{42}$ in the soluble and insoluble brain fractions and CSF and plasma were quantified by sensitive ELISA, as described.[41] Statistical comparisons were made with analysis of variance followed by Tukey post-hoc tests or Pearson's correlation. Power analyses indicate that we would be able to detect a 20% difference in tissue Aβ levels between groups before Aβ deposition (≤9 months) and a 60 to 70% difference between groups with deposition (eg, 15 months) using 10 to 15 animals per group. Thus, non-statistical differences in Aβ levels are interpreted as indicating differences less than 20% for young animals and 60% for older animals.

Western Blot

SDS-PAGE and Western blotting were performed as described.[42] Blots of mouse plasma were incubated with rabbit anti-mouse apoAI antibodies (Biodesign International; Saco, ME), followed by HRP-conjugated goat anti-rabbit antibodies (BioRad; Hercules, CA). Signal was detected by chemiluminescence (SuperSignal West Pico Chemiluminescence Substrate, Pierce; Rockford, IL) and quantified by Kodak Image Station (Rochester, NY).

Gel Filtration Chromatography

Samples of plasma (250 μl) from $PDAPP^{+/-}$, $apoAI^{+/+}$ and $PDAPP^{+/-}$, $apoAI^{-/-}$ mice (12 months old, $n = 2$ each, fasted and non-fasted) were fractionated under non-denaturing conditions over tandem Superose-6 HR 10/30 columns (Amersham Biosciences; Piscataway, NJ) using a BioLogic Workstation (BioRad) as described.[43] Adjacent fractions were pooled and assayed for total cholesterol as described below.

Cholesterol Assay

Plasma from all animals and cortical brain lysates (homogenized in PBS containing protease inhibitors) from a subset of 9- to 12-month-old animals before Aβ deposition were assayed for total cholesterol (Amplex Red Cholesterol Assay Kit, Molecular Probes; Eugene, OR) as previously described[44] and normalized to tissue wet weight. Small tissue volumes prevented us from analyzing both Aβ and cholesterol in the same hippocampal region, so another region known to exhibit Aβ deposition (parietal cortex) was chosen for cholesterol measures. Tissue homogenates included both soluble and insoluble (eg, membrane) fractions. Statistical comparisons between groups were made as described above.

Mouse apoE ELISA

Plasma from 15-month-old animals and brain tissue from 9-month-old animals before Aβ deposition were assayed for endogenous mouse apoE expression by an ELISA developed in our lab. Briefly, brain tissue (parietal cortex) was sonicated for 3 seconds on ice in apoE ELISA lysis buffer (PBS containing 0.05% Tween and protease inhibitors) before centrifugation at 14,000 rpm for 15 minutes at 4°C. The supernatant was transferred to another tube and stored at −70°C until analyzed. For the apoE ELISA procedure, microtiter plates were coated overnight with a monoclonal mouse anti-apoE antibody that recognizes mouse apoE (WUE4[45]) at a concentration of 4.5 μg/ml in carbonate-coating buffer (35 mmol/L $NaHCO_3$, 16 mmol/L Na_2CO_3, 0.02% Na azide, pH 9.6), and then blocked with 1% dry milk in PBS for 2 hours at RT. ApoE standards (Swiss Webster mouse plasma estimated to contain 50 μg/ml apoE) and samples of plasma or brain lysate from $PDAPP^{+/-}$, apoAI mice were diluted in apoE ELISA sample buffer (PBS containing 0.025% Tween, 0.1% bovine serum albumin (BSA) and protease inhibitors), loaded onto blocked ELISA plates, and incubated for 4 hours at RT. Plates were then incubated overnight at 4°C in biotinylated goat anti-apoE antibodies (125 μg/ml; Calbiochem; San Diego, CA) in PBS containing 1% BSA and 0.1% Na azide, followed by a 2-hour incubation in Strep-Poly HRP (Pierce) at RT and color development in Slow TMB for ELISA (Sigma; St. Louis, MO). Plates were read at 650 nm and quantified via FL600 Fluorescence Reader (Bio-Tek; Winooski, VT). Plates were rinsed 5 to 8 times with PBS between each step, and all incubations were carried out with rotation. This assay is sensitive down to 1.5 ng apoE/ml. ApoE levels in brain lysates were normalized to total protein levels, as measured by bicinchoninic acid (BCA) assay (Pierce). Statistical comparisons between groups were made as described above. Power analyses indicate an ability to detect differences of ≥60% between groups given the relatively small number of animals ($n = 5$) in each group.

Results

Total Cholesterol Levels in Plasma and Brain of $PDAPP^{+/-}$ Mice Are Significantly Reduced in the Absence of apoAI

The goal of the present study was to create a mouse model that develops AD-like pathology (ie, cerebral amyloidosis) and has variable levels of plasma cholesterol without the use of non-physiological dietary or pharmacological interventions. Consistent with previous studies of $apoAI^{-/-}$ mice,[31,32] $PDAPP^{+/-}$ mice lacking the endogenous mouse apoAI gene exhibited significant reductions (mean, 77%) in plasma cholesterol levels (Figure 1A) at all ages analyzed. Levels within groups did not differ as a function of age (data not shown). Isolation of plasma lipoproteins from $PDAPP^{+/-}$, $apoAI^{+/+}$ and $PDAPP^{+/-}$, $apoAI^{-/-}$ mice via size exclusion chromatography confirmed that this reduction was due to a marked decrease in plasma high density lipoprotein (HDL), the primary plasma lipoprotein in mice (Figure 1B), although decreases were also observed in very low density lipoprotein (VLDL) and low density lipoprotein (LDL). Fasting did not alter this pattern (data not shown). Thus we were successful in creating an animal model of AD-like

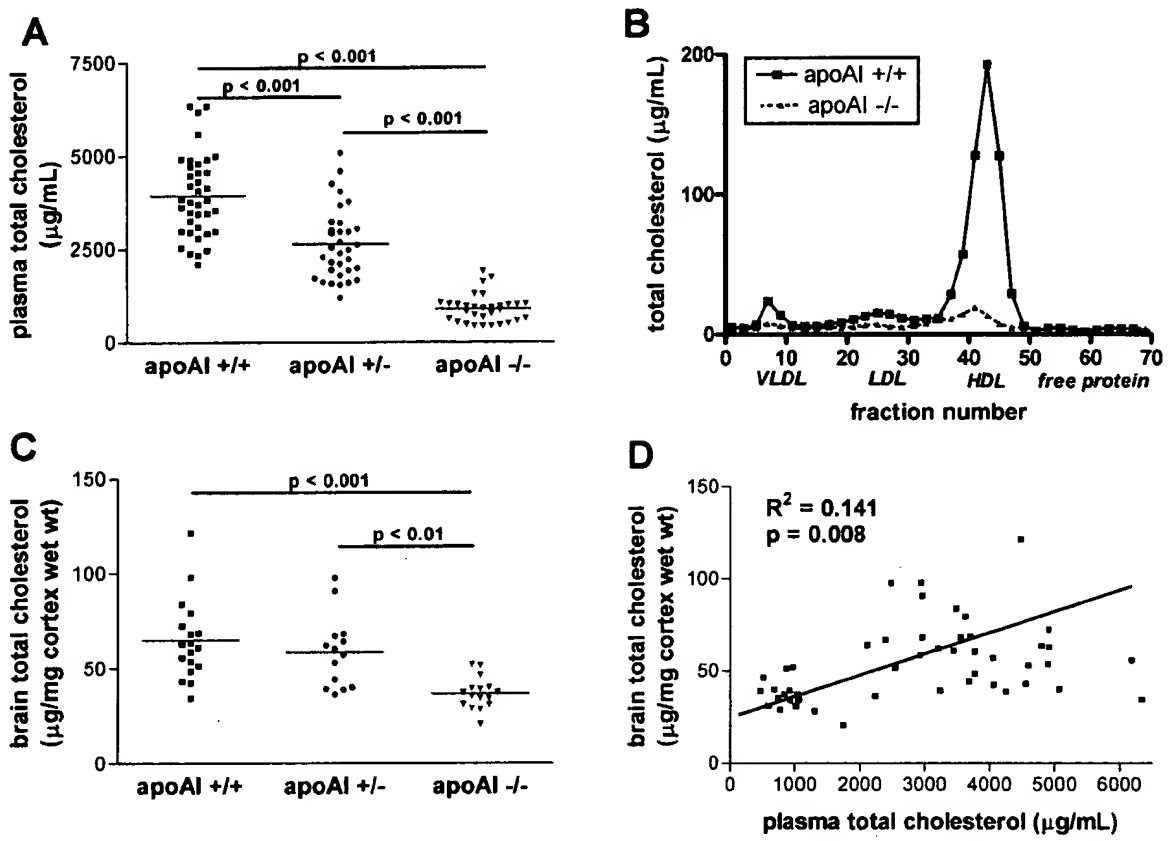

Figure 1. Effects of apoAI gene dose on plasma and brain lipid profiles in PDAPP$^{+/-}$ mice. **A:** Mean plasma total cholesterol levels significantly differ as a function of apoAI genotype in a gene dose-dependent manner (apoAI$^{+/-}$, 33% decrease compared to apoAI$^{+/+}$; apoAI$^{-/-}$, 77% decrease). **B:** Representative fractionation profile of plasma from 12-month-old PDAPP$^{+/-}$, apoAI$^{+/+}$ and PDAPP$^{+/-}$, apoAI$^{-/-}$ mice via gel filtration chromatography demonstrates a virtual absence of plasma HDL (the primary plasma lipoprotein in mice), as well as decreases in plasma VLDL and LDL in apoAI$^{-/-}$ mice. Total plasma cholesterol level in apoAI$^{+/+}$ = 4827 μg/ml. Total plasma cholesterol level in apoAI$^{-/-}$ = 318 μg/ml. **C:** Absence of apoAI (apoAI$^{-/-}$) also results in a significant decrease (43%) in mean total cholesterol levels measured in the brain (parietal cortex) of PDAPP$^{+/-}$ mice (9 to 12 months of age). **D:** Levels of brain total cholesterol are positively correlated (Pearson correlation) with levels of plasma total cholesterol in PDAPP$^{+/-}$, apoAI mice. HDL, high density lipoproteins; LDL, low density lipoproteins; VLDL, very low density lipoproteins.

cerebral amyloidosis that markedly differed in its level of plasma cholesterol (predominantly HDL).

Interestingly, we also observed significant reductions in cortical brain cholesterol levels in PDAPP$^{+/-}$, apoAI$^{-/-}$ mice compared to PDAPP$^{+/-}$, apoAI$^{+/+}$ mice (Figure 1C), although the magnitude of the difference was not as dramatic as was seen in plasma. There was ~40% less total brain cholesterol measured in PDAPP$^{+/-}$, apoAI$^{-/-}$ mice compared to PDAPP$^{+/-}$, apoAI$^{+/+}$ mice, although there was overlap between the groups. Brain cholesterol levels were significantly correlated with plasma cholesterol levels (Figure 1D). However, we observed no differences in the level of cholesterol in the CSF of apoAI$^{-/-}$ mice compared to C57BL/6 controls (17.7 ± 1.26 μg/ml in C57BL/6; 17.02 ± 0.49 μg/ml in apoAI$^{-/-}$, $P > 0.05$, mean ± SEM), nor significant differences between CSF apoE level in these animals (618 ± 304 ng/ml apoE in C57BL/6; 863 ± 240 ng/ml in apoAI$^{-/-}$, $P > 0.05$, mean ± SEM). To the extent that CSF reflects the composition of brain extracellular fluid, these data suggest that the reduction in brain total cholesterol we observed in PDAPP/apoAI$^{-/-}$ mice is due to changes in lipid pools other than lipoproteins in brain extracellular fluid. This could represent changes in brain cellular pools or could conceivably be related to residual plasma cholesterol associated with brain vasculature that is possibly not removed with standard systemic perfusion methods. Together these data suggest that apoAI, a protein produced predominantly by cells of the periphery (liver and intestine) and not the CNS (except perhaps by brain endothelial cells[46,47]), in some way influences cholesterol levels measured in the CNS, either through direct effects of apoAI on the brain or perhaps through interactions between cholesterol and/or lipoproteins in the plasma and the brain.

Reduction in Plasma Cholesterol Level Has No Effect on Age-Dependent Increases in Soluble or Insoluble $A\beta_{40}$ and $A\beta_{42}$ in the Hippocampus of PDAPP$^{+/-}$ Mice

Results from cell culture experiments[17–19] and *in vivo* models of pharmacological or dietary induced hypo- or hypercholesterolemia, respectively,[19–24] suggest a role for cholesterol in APP processing and Aβ generation. To directly test whether non-dietary and non-pharmacologi-

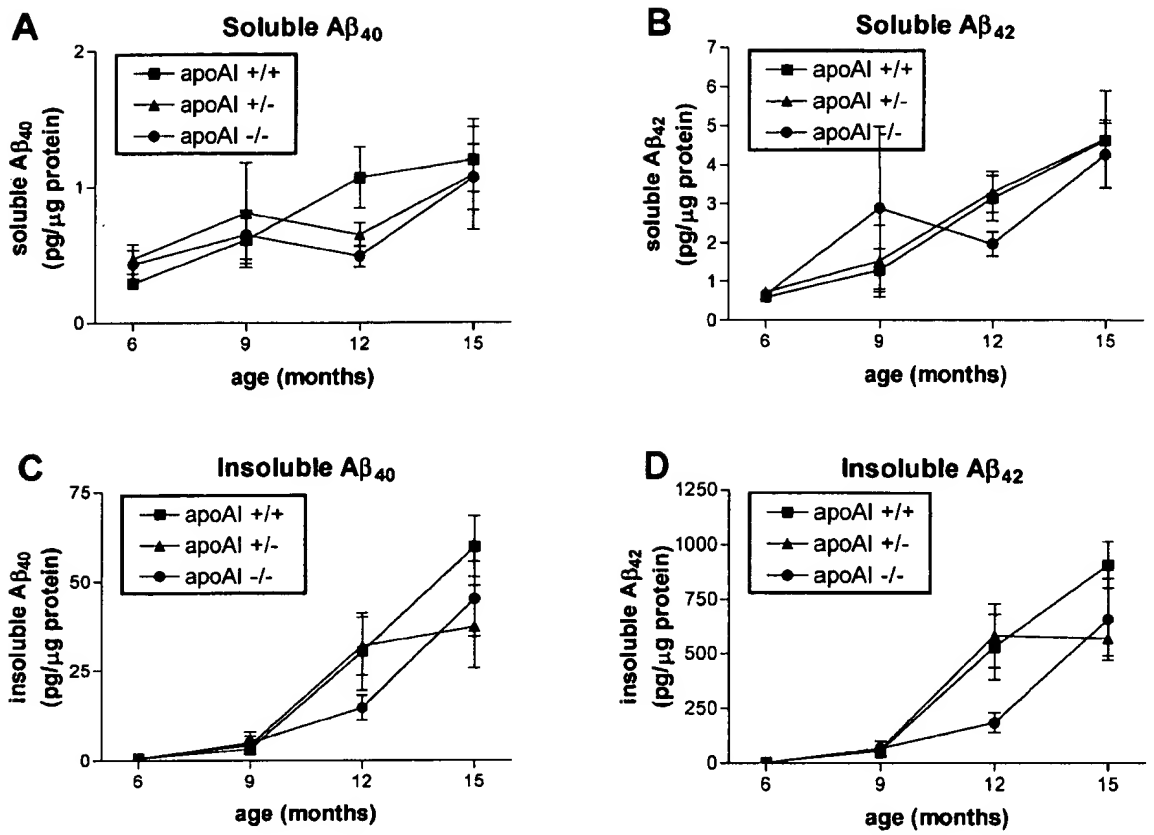

Figure 2. Levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus of PDAPP$^{+/-}$, apoAI mice with age. Levels of soluble (**A**) $A\beta_{40}$ and (**B**) $A\beta_{42}$ and insoluble (**C**) $A\beta_{40}$ and (**D**) $A\beta_{42}$ increase with age in PDAPP, apoAI mice, but do not differ significantly as a function of apoAI genotype. Values correspond to means ± SEM, 6 months, n = 2 to 3 animals per group; 9 months, n = 9 to 12 animals per group; 12 months, n = 9 to 14 animals per group; 15 months, n = 9 to 10 animals per group.

cal variations in plasma cholesterol levels influence brain Aβ levels, PDAPP$^{+/-}$, apoAI$^{+/+}$ (mean plasma cholesterol ± SEM = 3931 μg/ml ± 180), PDAPP$^{+/-}$, apoAI$^{+/-}$ (mean plasma cholesterol ± SEM = 2631 μg/ml ± 166), and PDAPP$^{+/-}$, apoAI$^{-/-}$(mean plasma cholesterol ± SEM = 896 μg/ml ± 68) mice were sacrificed at various ages, and the hippocampus was assayed for human $A\beta_{40}$ and $A\beta_{42}$ in the carbonate-soluble and carbonate-insoluble (guanidine-soluble) fractions. Consistent with previous reports of total Aβ (soluble plus insoluble),[48,49] levels of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus of PDAPP mice increased with age (Figure 2). These increases were all statistically significant ($P < 0.001$) except for soluble $A\beta_{40}$ ($P = 0.07$). Levels of insoluble $A\beta_{42}$ increased between 500- to 1000-fold from 6 to 15 months of age in all genotype groups. However, despite significant reductions in plasma and brain cholesterol levels (by 77% and 43%, respectively) with the absence of apoAI, the amount of soluble and insoluble $A\beta_{40}$ and $A\beta_{42}$ in the hippocampus and the time course of its increase did not differ between the genotype groups (Figure 2), nor was there a significant genotype by age interaction. Although levels of insoluble $A\beta_{40}$ and $A\beta_{42}$ in PDAPP$^{+/-}$, apoAI$^{-/-}$ mice were lower than the apoAI-expressing groups at 12 months of age (Figure 2, C and D), this difference was not observed in younger animals (6 to 9 months old) and values were not statistically different between the genotypes at older ages (15 months old). Consistent with the above findings, we observed no correlations between the level of brain cholesterol and any of the hippocampal Aβ levels (data not shown). In addition, levels of $A\beta_{40}$ and $A\beta_{42}$ in the CSF and plasma did not differ between the genotype groups (data not shown). These data demonstrate that life-long, non-dietary and non-pharmacological variations (up to fourfold) in the level of plasma cholesterol do not significantly influence steady-state Aβ levels in the CNS or plasma of PDAPP mice.

The Amount, Pattern, and Age of Onset of Plaque Deposition in PDAPP$^{+/-}$ Mice Does Not Differ as a Function of Plasma Cholesterol Levels Due to apoAI Genotype

Since cholesterol accumulates in senile plaques in AD brain and APP transgenic mice,[12] binds to Aβ[50] and promotes Aβ fibril formation,[51] we next investigated whether the reductions in plasma cholesterol observed in PDAPP$^{+/-}$, apoAI$^{-/-}$ mice had any effect on the deposition of Aβ as diffuse or amyloid plaques. PDAPP$^{+/-}$ mice of the different apoAI genotypes were sacrificed at 6, 9, 12, and 15 months of age, and the amount of Aβ and

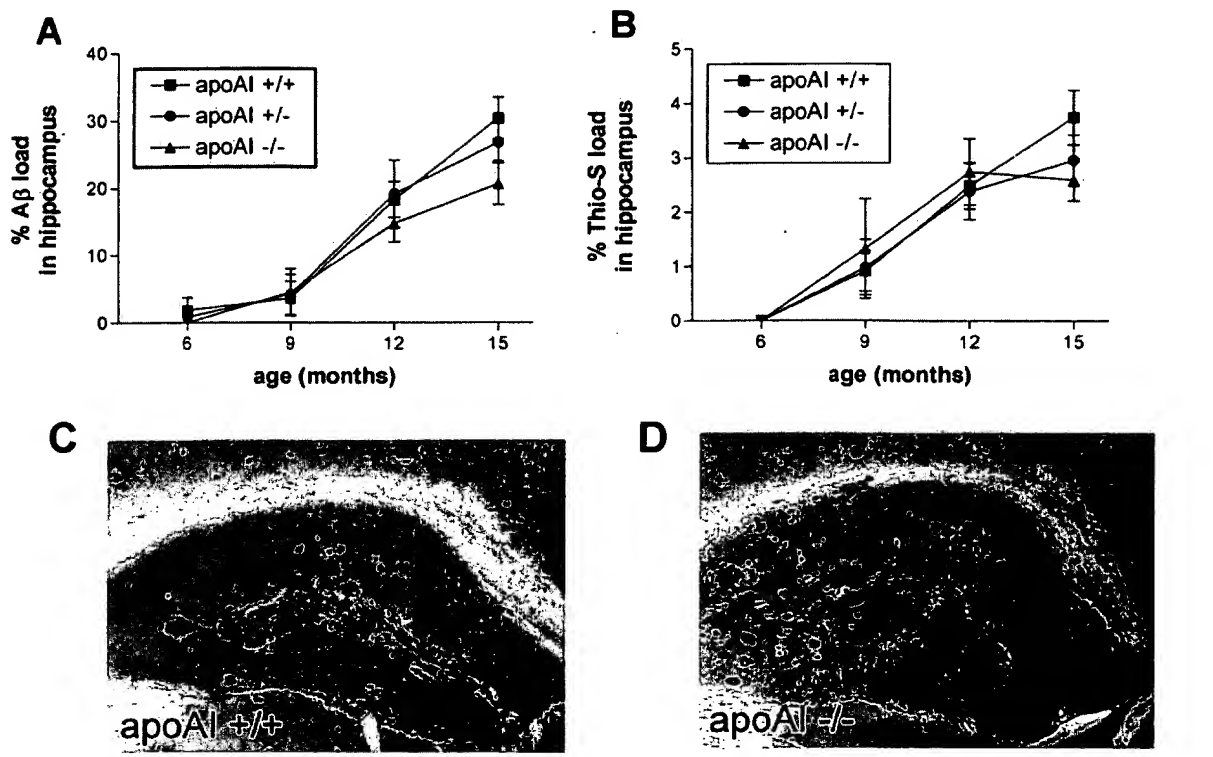

Figure 3. Aβ deposition in the hippocampus of PDAPP mice as a function of apoAI genotype. **A:** Aβ load (defined as the percentage of hippocampal area in three tissue sections covered by Aβ immunoreactivity) and (**B**) amyloid load (defined as the percentage of hippocampal area in three tissue sections covered by Thioflavine-S positivity) increases with age in PDAPP$^{+/-}$, apoAI mice, but do not differ significantly as a function of apoAI genotype. Aβ plaques are observed in similar patterns throughout the hippocampus and overlying cortex in both (**C**) PDAPP$^{+/-}$, apoAI$^{+/+}$ and (**D**) PDAPP$^{+/-}$, apoAI$^{-/-}$ mice (12 months of age). Values correspond to means ± SEM, 6 months, n = 2 to 3 animals per group; 9 months, n = 9 to 12 animals per group; 12 months, n = 9 to 14 animals per group; 15 months, n = 9 to 10 animals per group.

amyloid deposition in the hippocampus was quantified by unbiased, stereologic methods. In agreement with previous reports,[48,49] Aβ deposition in PDAPP$^{+/-}$ mice (wild-type for the apoAI gene) increased with age (Figure 3A). The amount and age of onset of Aβ deposition, however, did not differ significantly between the apoAI genotype groups (Figure 3A), nor did the pattern of plaque distribution within the hippocampus (Figure 3, C and D). There were also no significant differences between the numbers of amyloid plaques, as defined by staining with Thioflavine-S (Figure 3B), nor in the amount of neuritic dystrophy associated with amyloid plaques, as assessed by the de Olmos silver stain (data not shown). Consistent with these findings, we observed no correlation between plasma (R^2 = 0.006, P = 0.64) or brain (R^2 = 0.045, P = 0.24) cholesterol levels and hippocampal Aβ deposition. Thus, dramatic reductions in plasma cholesterol secondary to the absence of apoAI does not appear to influence Aβ levels or deposition in this mouse model.

ApoE Expression Is Increased in the Plasma But Not the Brain of PDAPP$^{+/-}$ Mice Lacking apoAI

Given the finding of a lack of effect of plasma cholesterol on Aβ-related pathology in this animal model, we quantified the expression of another apolipoprotein, apoE, in the brain and plasma of PDAPP mice of the different apoAI genotypes before Aβ deposition. ApoE is normally expressed in both the brain and the periphery, but its levels are regulated independently in these two compartments.[16,52] Furthermore, apoE is known to exert profound effects on Aβ fibrillogenesis,[53,54] and Aβ metabolism in human AD[55] and mouse models of AD-like cerebral amyloidosis in a dose-dependent fashion.[35,37,49,56–58] ApoE levels in plasma (15 months old) and homogenates of parietal cortex (9 months old without Aβ deposition) from animals of each genotype were quantified by a sensitive ELISA. We observed a marked increase in apoE levels in the plasma of PDAPP$^{+/-}$ mice lacking apoAI, consistent with previous studies of apoAI$^{-/-}$ mice[59,60] (Figure 4A). Interestingly, however, there was no significant difference in apoE levels in the brain between any of the apoAI genotype groups (Figure 4B). Our combined observations of equivalent Aβ pathology in animals with equal expression of brain apoE but reduced levels of cholesterol are consistent with the hypothesis that it is perhaps the level of apoE, and not cholesterol *per se*, that influences Aβ metabolism in this mouse model.

Discussion

Results of the present study demonstrate that absence of apoAI, the major plasma HDL-associated apolipoprotein, leads to marked (mean, 77%) reductions in plasma cho-

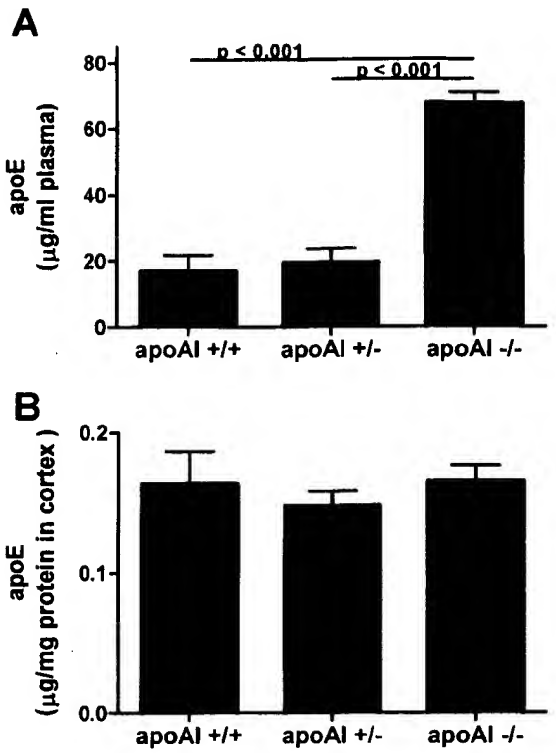

Figure 4. ApoE protein levels in the plasma and brain of PDAPP$^{+/-}$ mice as a function of apoAI genotype. **A:** ApoE level in the plasma of PDAPP$^{+/-}$, apoAI$^{-/-}$ mice is significantly greater than that of PDAPP$^{+/-}$, apoAI$^{+/+}$ and PDAPP$^{+/-}$, apoAI$^{+/-}$ mice (all 9 months old without Aβ deposition). **B:** ApoE levels in the brain of PDAPP$^{+/-}$, apoAI mice do not differ as a function of apoAI genotype. Values correspond to means ± SEM, n = 5 to 6 animals per group.

lesterol levels in PDAPP$^{+/-}$ mice. Hence, we were able to use this genetic model to directly test whether non-dietary, non-pharmacological variations in plasma cholesterol level influence brain Aβ levels and deposition, a hypothesis proposed to explain the reported link between high plasma cholesterol and increased risk for AD.[61,62] Interestingly and in contrast to animal models in which non-physiological high fat diets or pharmacological means are used to modify plasma cholesterol levels, we observed no differences in the age-related development, pattern or extent of Aβ-related pathology in PDAPP mice of the various apoAI genotypes despite up to fourfold differences in normal plasma cholesterol levels between the groups. The absence of apoAI also resulted in reduced levels of cholesterol measured in the brain (mean, 43% reduction) but not CSF, but had no effect on CNS apoE levels. Together, these data are consistent with the idea that it is the level of brain apoE, not plasma cholesterol *per se*, which influences Aβ metabolism and its deposition in the brain.

Low HDL cholesterol is a known risk factor for coronary artery disease,[63,64] perhaps by impairing reverse cholesterol transport capability. ApoAI is the major apolipoprotein associated with HDL, and apoAI deficiency in humans leads to a phenotype of low plasma HDL levels and premature atherosclerosis.[65–67] ApoAI knockout mice also exhibit a marked reduction in plasma HDL levels[31,32] that is reflected in levels of total plasma cholesterol since HDL is the primary plasma lipoprotein in mice. Interestingly, apoAI$^{-/-}$ mice do not develop atherosclerosis,[60] although they have been reported to exhibit diminished HDL cholesteryl ester flux and tissue uptake of HDL cholesteryl esters.[32] However, HMG-CoA reductase activity (important for cholesterol biosynthesis) and LDL receptor levels are normal in apoAI$^{-/-}$ mice (except in steroidogenic tissues), as are cholesterol and cholesteryl ester stores in a variety of tissues examined.[32] Cholesterol levels in the brain with apoAI deficiency have not been examined. We observed reduced levels of cholesterol in brain but not in CSF in mice lacking apoAI. Although our methods at the time did not permit assessment of the different pools of cholesterol in brain (ie, free versus esterified cholesterol), more recent experiments using tissue from various mouse strains (including apoAI-null mice) has demonstrated that brain contains predominantly (>95%) free (non-esterified) cholesterol (S. Wahrle, unpublished observations). Therefore, it is free cholesterol that is most likely decreased in PDAPP/apoAI$^{-/-}$ mice.

The observation of reduced levels of brain cholesterol in PDAPP/apoAI$^{-/-}$ mice may indicate a direct or indirect effect of apoAI on brain cholesterol metabolism or alternatively may reflect plasma HDL cholesterol associated with brain vasculature that is possibly not removed by systemic perfusion. ApoAI is synthesized primarily by cells of the liver and intestine[68,69] but is found in brain homogenates,[70] perhaps a product of brain endothelial cells,[46,47] and in CSF,[16,71,72] as a presumed filtrate of plasma. Thus, to the extent that apoAI can enter brain parenchyma from the plasma and CSF, apoAI could conceivably interact directly with neural tissue elements and modify local cholesterol metabolism. The cellular (eg, neurons or glia) or subcellular (eg, myelin, lipid rafts, interstitial fluid) origins of the observed brain cholesterol deficit in PDAPP, apoAI$^{-/-}$ mice remain to be determined. In general, the cellular and molecular mechanisms governing cholesterol metabolism in the CNS are poorly understood and are likely complex. Indeed, the overlap in brain cholesterol levels observed between the apoAI genotype groups suggests that molecules in addition to apoAI are involved in brain cholesterol metabolism. The fact that CSF cholesterol did not differ between wild-type and apoAI$^{-/-}$ mice suggests that brain extracellular lipoprotein metabolism is not affected by apoAI deficiency. As mentioned above, while our methods of quantifying cholesterol in tissue are very sensitive and reproducible, the possible contribution of residual plasma HDL cholesterol that remains associated with brain vasculature after systemic perfusion has not been defined. Thus, the changes in total brain cholesterol in PDAPP/apoAI$^{-/-}$ mice may not be due to changes in neuronal or glial cholesterol but may possibly reflect vascular cholesterol of a plasma origin. This issue will need to be addressed in future studies.

Reduced brain cholesterol levels in the absence of apoAI may alternatively indicate indirect actions of apoAI on the brain, secondary to reductions in plasma HDL and total cholesterol levels. Although regulation of brain cho-

lesterol metabolism has long been considered to be independent of that in plasma, we have recently reported a strong positive correlation between the level of CSF lipoproteins (known to be HDL-like) and plasma HDL, but not LDL, in cognitively normal elderly individuals.[16] Furthermore, a positive correlation was observed between the level of apoAI, but not apoE, in CSF and plasma, suggesting a possible role of plasma apoAI/HDL in modulating CNS lipoprotein metabolism.[16] Interestingly, decreased HDL and plasma apoAI concentrations have been reported to correlate highly with the severity of dementia in AD.[73] Whether other diseases that lead to reduced plasma HDL levels (eg, apoAI mutations or Tangier's disease) affect CNS cholesterol levels or influence AD risk has not been reported.

The absence of an Aβ phenotype in PDAPP, apoAI$^{-/-}$ mice was somewhat surprising given data supporting a role for cholesterol in influencing AD risk and Aβ metabolism. However, a closer inspection of the published data point to a possible reason for this discrepancy and, perhaps more importantly, allows for an alternative interpretation of the published data that is consistent with the present results. In human and animal studies, hyper- and hypocholesterolemia induced by high fat diets and use of the cholesterol-lowering drugs known as statins, respectively, are also associated with alterations in brain apoE levels. High fat diets not only increase the level of cholesterol, but also apoE, in the brain,[20,21,74] and statins decrease them both.[75,76] Thus, it is not possible to distinguish putative effects of cholesterol from those of apoE on brain Aβ metabolism in these studies. Indeed, it is conceivable that effects of high fat diets and statin treatment previously attributed to cholesterol are actually due to altered levels of brain apoE. Consistent with this idea are studies showing that cholesterol effects on APP processing appear to require the presence of apoE,[21] and lovastatin treatment influences brain cholesterol levels in wild-type mice but has no effect in apoE$^{-/-}$ mice.[77] Our present finding of no alterations in Aβ-related measures in PDAPP, apoAI$^{-/-}$ mice in the presence of reduced plasma and brain cholesterol levels but equivalent levels of brain apoE would thus be consistent with this proposed primary role of apoE, rather than cholesterol, in brain Aβ metabolism *in vivo*. ApoE is known to exert profound effects on Aβ fibrillogenesis *in vitro*[53,54] and on Aβ deposition in human AD.[55] Murine and human apoE have also been shown to have marked dose-dependent effects on Aβ fibrillogenesis, clearance, and toxicity *in vivo* in mouse models of AD-like cerebral amyloidosis.[35,37,49,56–58] It is particularly noteworthy that apoE$^{-/-}$ mice have extremely high levels of plasma cholesterol associated with VLDL and normal levels of brain cholesterol,[44] yet mouse models of amyloidosis lacking apoE display significant reductions in Aβ deposition, especially deposits that are true amyloid (ie, Thioflavine-S positive).[35,37] This dissociation strongly argues that the main effect of apoE on Aβ metabolism is not obviously linked with total brain or plasma cholesterol but is much more likely due to its direct effect as an Aβ chaperone.

Together, our findings suggest that the reported link between plasma cholesterol metabolism and AD pathogenesis may be due to mechanisms other than, or in addition to, direct effects of cholesterol on Aβ metabolism, and further strengthen the idea that regulating the level of brain apoE may be an important therapeutic approach for AD treatment. Studies aimed at directly modifying apoE level in the brain (independent of cholesterol) in mouse models, for example through gene transfer approaches, are currently in progress to test this hypothesis.

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